HEMOGLOBIN SYNTHESIS IN RABBIT RETICULOCYTES IN VITRO*

By JACQUES KRUH† and HENRY BORSOOK

(*From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California*)

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We have reported the rapid incorporation of labeled amino acids into the proteins of rabbit reticulocytes *in vitro* and the stimulation of this process by certain amino acids, iron, fructose-amino acids, glucose, and some, as yet, unidentified material in the filtrate of boiled plasma. The incorporation was measured in the mixture of the total proteins of the reticulocytes (1, 2).

In the study reported here two protein fractions were isolated, the hemoglobin, which comprises more than 75 per cent of the total, and the water-insoluble proteins. The hemoglobin was fractionated into heme and globin. Experiments were carried out with four C^{14}-labeled amino acids and with different stimulators to ascertain whether heme synthesis and amino acid incorporation went parallel or not.

The four C^{14}-amino acids used were methylene-labeled glycine and carboxyl-labeled L-histidine, L-leucine, and L-lysine. Under the conditions of our experiments, significant radioactivity in the heme was to be expected when methylene-labeled glycine was used, but none with the labeled histidine, leucine, or lysine, or with carboxyl-labeled glycine (3). This was confirmed in the first experiments with each amino acid. Thereafter, the isotopic glycine used was always methylene-labeled, and the radioactivity of the heme was measured only in the experiments with glycine so labeled. The resulting radioactivity of the globin and of the water-insoluble proteins was, of course, measured in all the experiments.

Labeled Amino Acids—Methylene-labeled C^{14}-glycine was obtained from Tracerlab, Inc.; as used its specific activity was 5.5×10^6 c.p.m. per mmole. Syntheses of L-histidine, L-leucine, and L-lysine were as previously described (1, 4, 5); their specific activities were, respectively, 2.31, 2.29, and 1.56 $\times$

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† French Government and Fulbright Fellow. Present address, Laboratoire de Recherches de Biochimie Médicale, Hôpital des Enfants Malades, 149 rue de Sevres, Paris 15, France.

10^6 c.p.m. per mmole. Their initial concentrations in the reaction mixture were always 0.001 M.

Unlabeled Amino Acid Mixture

The reaction mixture contained the following unlabeled amino acids, concentrations being expressed as micrograms per ml. of reaction mixture: L-arginine 14.8, L-aspartic acid 25.1, glycine 11.0, L-histidine 21.6, L-isoleucine 9.7, L-leucine 25.0, L-lysine 41.0, L-phenylalanine 28.0, DL-serine 46.0, L-threonine 13.5, L-tryptophan 4.5, and L-valine 24.3. The unlabeled amino acid corresponding to the labeled amino acid used in the experiment was omitted.

Iron—Iron was added usually as $FeSO_4(NH_4)_2 \cdot SO_4 \cdot 6H_2O$. Ferrous chloride was equally effective but less convenient. To obtain consistent and maximal effects it was necessary to add the iron to the reaction mixture after the reticulocytes as the last ingredient before incubation.

Fructose-Amino Acids—Four fructose-amino acids (1-deoxy-1-amino-2-ketohexoses) (2, 6), *i.e.* those of L-aspartic acid, glycine, L-leucine, and L-phenylalanine, were used, each at 2×10^{-4} M, which is approximately the minimal concentration giving maximal stimulation with 0.5 γ of Fe per ml. To obtain the stimulating action of fructose-amino acids, it is necessary to add the above unlabeled amino acid mixture to the reaction mixture.

Plasma Filtrate—The plasma filtrate was prepared from the blood of rabbits made anemic with phenylhydrazine as previously described (1). This filtrate still contains some protein; when it is dialyzed against water until the dialysate no longer gives a test with $AgNO_3$, most of the stimulating activity is found in the dialysate. In some of the later experiments the dialysate was used instead of the whole plasma filtrate.

Reaction Mixture and Incubation Procedure

The production of the reticulocytosis and the preparation of the reticulocytes were as described previously (1). The incubation was carried out in 20 ml. beakers in the Dubnoff apparatus (7) under 95 per cent O_2 and 5 per cent CO_2 at 37.5°, for either 2 or 4 hours as indicated below, with a rocking rate of 100 cycles per minute. Each beaker contained 1 ml. of packed reticulocytes in 8 ml. of reaction mixture; the solvent was Krebs-Henseleit solution (8). As six to twelve such beakers were needed to provide enough heme and water-insoluble protein for accurate counting, and the Dubnoff apparatus holds only thirty beakers, it was possible in any one experimental run to compare, at the most, only four variants. Incorporation of glycine was the common reference from experiment to experiment. This choice was fortunate, because the rates of heme synthesis and of the incorporation of glycine per residue of glycine in the globin turned out to be the same.

All the glassware and all the solutions except the amino acid mixture were sterilized in the autoclave. Since repeated autoclaving was found to destroy phenylalanine, the amino acid mixture was boiled once and then kept in the deep freeze in small flasks with just enough, usually 30 ml., for one experiment per flask.

At the end of the incubation the cells were washed five times by suspending and then centrifuging in 10 volumes of Krebs-Henseleit solution each time. The cells were then hemolyzed with water.

Preparation of Hemoglobin

The hemoglobin was isolated and purified by a modification of the method of Roche *et al.* (9–11). Equivalent amounts of KH_2PO_4 and K_2HPO_4 were added to give 2.9 M total phosphate. All the plasma and cellular proteins were salted out, whereas rabbit hemoglobin, which is extremely soluble, remained in solution. After standing overnight at room temperature, the salted out proteins were removed by filtration. The hemoglobin was then salted out by further addition to the filtrate of equivalent amounts of the two phosphate salts to give 3.5 M phosphate. The hemoglobin was collected by filtration on a Büchner funnel and washed on the filter with 3.5 M phosphate solution. The washed hemoglobin was then dissolved in about 50 ml. of water and dialyzed at 4° for 3 days against repeated changes of water (total volume 10 liters).

Preparation of Heme—The heme was isolated from the hemoglobin by an adaptation of the method of Anson and Mirsky (12). 10 volumes of 1 per cent HCl in acetone were added to the hemoglobin solution; the globin, which precipitated, was removed by filtration, an equal volume of water was added to the filtrate, and the diluted acetone solution was evaporated under a lamp.[1] After a few hours the heme precipitated; it was washed by decantation four times with water, dried overnight at 100°, dissolved in 0.5 ml. of pyridine, and then filtered onto a circle of lens paper that fitted exactly in a circular aluminum cup, 19 mm. in diameter, and dried under a lamp. With 8 to 15 mg. of heme, a uniform spread was obtained for the measurement of the radioactivity.

Preparation of Globin—The globin which had been filtered from the heme was washed on the filter with 1 per cent HCl in acetone until the washings were colorless. It was then suspended in 80 ml. of 7 per cent trichloroacetic acid, set away at room temperature overnight, centrifuged, redissolved in 2 ml. of 1 N NaOH, and then reprecipitated by 100 ml. of 7 per cent trichloroacetic acid. The precipitated protein was transferred to a centrifuge tube, in which it was washed twice with 7 per cent trichloroacetic acid, twice with a mixture of equal volumes of acetone and ether, once with

[1] Unpublished method of Dreyfus, J. C., Kruh, J., and Schapira, G.

acetone, twice with ether, and then dried at 85°. The globin thus obtained was a fine, white powder.

Preparation of Water-Insoluble Proteins—The insoluble material remaining after the thoroughly washed red cells had been lyzed was washed thoroughly with water to remove all hemoglobin and other soluble proteins. It was then suspended in 100 ml. of 7 per cent trichloroacetic acid and heated for 10 minutes at 90° to remove nucleic acids. From this point on the procedure was the same as that with globin.

Measurement of Radioactivity—The material whose radioactivity was to be measured was spread uniformly on an aluminum plate 19 mm. in diameter and counted in a Geiger-Müller end window counter (13). An empirical self-absorption curve was used to correct for the thickness of the sample.

TABLE I

Stimulation of Heme Synthesis and Amino Acid Incorporation by Iron

In the blanks the degree to which the labeled amino acid was incorporated into the globin varied with different batches of cells from 2 to 8 mmoles per mole of that amino acid in globin. No results with iron but without the amino acid mixture are given because added iron has an insignificant effect without the addition of the amino acids. The results are expressed as per cent of the blank value. Incubation, 4 hours. In Tables I, II, III, and V, Gly = glycine, His = histidine, Leu = leucine, Lys = lysine.

	Heme synthesis	Amino acid incorporation into							
		Globin				Water-insoluble proteins			
		C^{14}-Amino acid used							
	Gly	Gly	His	Leu	Lys	Gly	His	Leu	Lys
Blank (amino acid mixture alone)	100	100	100	100	100	100	100	100	100
" + iron, 0.5 γ per ml.	112	142	123	137	140	80	101	129	121
" + " 5.0 " " "	196	180	156	150	188	78	121	167	188

Calculations

Heme—Heme-pyridine hemochromogen contains 2 residues of pyridine per heme (14). 8 molecules of glycine enter into the synthesis of 1 of heme (3). On this basis the number of millimoles of heme synthesized per $\frac{1}{4}$ molecule of hemoglobin (or per mole of heme attached to hemoglobin) isolated is given by ((counts per minute per mg. of heme-pyridine)/(counts per minute per millimole of labeled glycine × 8)) × 774 × 10^3.

Incorporation of Amino Acids into Globin—In order to compare the rates

of incorporation of four different amino acids into globin it is necessary to take into account the amounts of these amino acids in the globin. There are no reliable analytical data on rabbit hemoglobin. Accordingly, we have used the following data of Schroeder *et al.* (15) on human hemoglobin expressed as residues per molecule of hemoglobin: glycine 43.1, histidine 36.3, leucine 76.0, and lysine 44.0. Tristram (16) gives the following (as residues per molecule of hemoglobin) for horse hemoglobin: glycine 48, histidine 36, leucine 75, and lysine 38. In view of the similar amino acid composition

TABLE II

Stimulation of Heme Synthesis and Amino Acid Incorporation by Fructose-Amino Acids

In each case the fructose-amino acid was added to a concentration in the reaction mixture of 2×10^{-4} M. The results are expressed as per cent of the blank value. Incubation, 4 hours.

	Heme synthesis	Amino acid incorporation into							
		Globin				Water-insoluble proteins			
		C^{14}-Amino acid used							
	Gly	Gly	His	Leu	Lys	Gly	His	Leu	Lys
Blank (amino acid mixture alone)	100	100	100	100	100	100	100	100	100
" + iron, 0.5 γ per ml.	112	142	121	142	140	80	129	101	120
" + " 0.5 " " " + fructose-L-aspartic acid	164	215				59			
Blank + iron, 0.5 γ per ml. + fructose-glycine	180	230	140	198	170	54	167	129	121
Blank + iron, 0.5 γ per ml. + fructose-L-leucine				190	161		179		109
Blank + iron, 0.5 γ per ml. + fructose-L-phenylalanine	152	234		211	173	56	175		120

of human and horse hemoglobins it seems likely that the values obtained by Schroeder *et al.* for human hemoglobin can be used for rabbit hemoglobin for purposes such as ours, without significant error.

The number of millimoles of labeled amino acid incorporated into hemoglobin per residue of that amino acid in the protein, let glycine be an example, is given by ((counts per minute per mg. of globin)/(counts per minute per millimole of glycine $\times$ 43.1)) $\times$ 6.6 $\times$ 10^7.

Incorporation of Amino Acids into Water-Insoluble Proteins—The water-insoluble proteins are a mixture of undetermined composition. The rate of incorporation was expressed as micromoles per gm. of protein, which is

given by ((counts per minute per mg. of protein)/(counts per minute per millimole of labeled amino acid)) $\times 10^6$.

Results

Tables I, II, and III show the stimulation, respectively, by iron, fructose-amino acids, and plasma filtrate. All three of these stimuli required addition of the amino acid mixture to be effective, and the last two also required iron. The stimulating effect of the fructose-amino acids is clear with 0.5 γ of iron per ml., but, when added with 5.0 γ of iron per ml., there is little or no more stimulation than by this amount of iron alone; the effect of the plasma filtrate is superimposed on that of a high concentration of iron. The synthesis of heme and the incorporation of the four amino acids

TABLE III

Stimulation of Heme Synthesis and Amino Acid Incorporation by Plasma Filtrate

The results are expressed as per cent of the blank value. Incubation, 4 hours.

	Heme synthesis	Amino acid incorporation into							
		Globin				Water-insoluble proteins			
		C^{14}-Amino acid used							
	Gly	Gly	His	Leu	Lys	Gly	His	Leu	Lys
Blank (amino acid mixture alone)	100	100	100	100	100	100	100	100	100
" + iron, 5.0 γ per ml.	165	183	175	228	183	72	150	180	169
" + " 5.0 " " " + plasma filtrate, 0.25 ml. per ml	298	298	300	320	251	59	249	220	203

into globin and that of histidine, leucine, and lysine into the water-insoluble proteins were all stimulated. The incorporation of glycine into the water-insoluble proteins was decreased by all three stimuli; we have no explanation for this effect

The rates of synthesis of heme and of glycine incorporation into globin per glycine residue were equal (Table IV). No systematic variation is discernible in the ratio of these two rates with different stimulating substances and a wide range of activity in the cells. This result would not have occurred if there had been a significant pool in the cells of unlabeled intermediates of heme or of globin, or of free heme or globin. The rates of synthesis of the two parts of hemoglobin must have been nearly the same.

A number of experiments were carried out to ascertain the relative rates of incorporation of several different amino acids into globin under different conditions of stimulation. The procedure was to add labeled glycine, histi-

TABLE IV

Comparison of Rate of Synthesis of Heme and Concurrent Incorporation of Glycine into Globin under Different Conditions of Stimulation and during Different Lengths of Time

Stimulating substances added to reaction mixture (1)	Duration of experiment (2)	No. of experiments in group (3)	Heme, mmoles synthesized per mole of total heme (4)*	Globin, mmoles glycine incorporated per glycine residue in globin (5)*	Ratio, (5)/(4) (6)†	(7)†
	hrs.					χ^2
Blank (amino acid mixture alone)	1	1	1.67	2.19	1.31	3.897
" "	2	1	2.48	3.31	1.33	4.414
" "	4	6	2.53–8.04	2.58–7.57	1.06	0.878
Blank + iron, 0.5 γ per ml.	2	3	2.92–6.60	2.81–5.86	0.95	0.304
" + " 1.0 " " "	2	5	3.16–3.88	2.66–3.54	0.88	2.938
" + " 5.0 " " "	4	1	4.57	5.12	1.12	0.584
" + " 0.5 " " " + fructose-aspartic acid	2	1	7.51	6.30	0.83	1.171
Blank + iron, 0.5 γ per ml. + fructose-aspartic acid	4	1	5.18	5.53	1.06	0.146
Blank + iron, 0.5 γ per ml. + fructose-glycine	2	1	6.64	5.94	0.89	0.490
Blank + iron, 0.5 γ per ml. + fructose-glycine	4	2	3.45–5.70	3.00–5.93	0.92	0.529
Blank + iron, 0.5 γ per ml. + fructose-histidine	2	1	2.63	2.82	1.07	0.198
Blank + iron, 0.5 γ per ml. + fructose-phenylalanine	2	2	2.84–4.83	2.92–6.04	1.13	1.394
Blank + iron, 1.0 γ per ml. + plasma filtrate, 0.25 ml. per ml.	2	3	4.87–5.60	4.49–5.78	0.96	0.197
Blank + iron, 5.0 γ per ml. + plasma filtrate, 0.25 ml. per ml.	1	1	2.87	2.80	0.97	0.036
Blank + iron, 5.0 γ per ml. + plasma filtrate, 0.25 ml. per ml.	4	2	5.68–9.36	7.48–10.33	1.20	3.305
Blank + liver extract, 0.5 ml. per ml.	2	4	3.54–5.40	3.22–5.44	0.90	1.643
Blank + liver extract, 0.5 ml. per ml. + plasma filtrate, 0.25 ml. per ml.	2	2	3.62–5.14	3.18–5.67	0.98	0.033
Blank + iron, 5.0 γ per ml. + fructose-alanine + plasma filtrate, 0.075 ml. per ml.	4	1	5.74	5.48	0.96	0.064
Blank + iron, 5.0 γ per ml. + fructose-glycine + plasma filtrate, 0.075 ml. per ml.	4	1	5.24	4.87	0.93	0.198
Average					1.00	
Standard deviation					±0.157	
χ^2						22.419
P						0.26

TABLE IV—*Concluded*

The fructose-amino acids were at a concentration of 2×10^{-4} M. Unlabeled L-serine was added to the reaction mixture equivalent to one-quarter of the total labeled glycine added. Serine is formed from glycine in reticulocytes. When no unlabeled serine was added, 14 per cent of the radioactivity in the globin after experiments with radioactive glycine was found to be due to radioactive serine (1). In these experiments the labeled serine formed from the glycine was diluted by the unlabeled serine in the reaction mixture. Accordingly, much less, probably less than 5 per cent, of the total radioactivity in the globin after the use of labeled glycine was due to labeled serine incorporated. No correction was applied. In the statistical treatment of the data, the hypothesis was that 1.00 was the correct value in Column 6. On this basis the standard deviation, σ, computed from the array of thirty-nine individual values of the ratio in Column 6, was 0.157. χ^2 for each group was computed by means of the formula $\chi^2 = \Sigma((\bar{x} - m)/(\sigma/\sqrt{n}))^2$, where $\bar{x}$ was the mean for the group of the observed values of the ratio in Column 6, $m = 1.00$ (by hypothesis), and n = the number of experiments in the group.

* Range of values.

† Average of individual values in the group.

dine, leucine, or lysine to different aliquots of the same cells containing, otherwise, the same reaction mixture, and then to incubate all the aliquots concurrently under identical conditions.

The results obtained are summarized in Table V. The amino acid incorporation was first calculated as millimoles of amino acid incorporated per mole of that amino acid in globin.[2] The ratios of the values of pairs of amino acids are given in Table V. They show that, per mole of the amino acid in globin, glycine, histidine, leucine, and lysine were incorporated at the same rate. All the deviations from 1 in the different sets of ratios are within the experimental error and those of the uncertainties of the correction factor applied to the glycine incorporation. A correction factor is needed for the specific activity of the glycine because the labeled glycine added was diluted to a significant extent by unlabeled glycine in the cells. The correction factor probably would vary from experiment to experiment, but would be within the range 1.3 to 1.6; 1.45 was used in the calculations of Table V.[3]

[2] We have found previously (1) that after the incorporation of C^{14}-labeled histidine, leucine, or lysine all of the radioactivity in the protein was accounted for by the incorporated labeled amino acid which was isolated as such. After the use of labeled glycine, 79 per cent of the radioactivity was found in the isolated glycine and 14 per cent in the serine. In the experiments of Table V the labeled serine that was formed from the 8 μmoles of labeled glycine initially in the reaction mixture was diluted by 1.8 μmoles of added unlabeled L-serine. Accordingly, we feel that it is warranted to ascribe in every case all the radioactivity in the globin to the incorporation of the labeled amino acid added to the reaction mixture.

[3] This estimate is based on the following considerations: The concentration of glycine in reticulocytes is about 3.2 to 7.5 mM (1, 17). The concentration of added labeled glycine was 1 mM, and the volume of the reaction mixture, containing 1 ml.

TABLE V

Comparison of Rates of Concurrent Incorporation into Globin of Glycine, Histidine, Leucine, and Lysine

The ratios were those of millimoles of amino acid incorporated per mole of that amino acid in globin. The degree of incorporation so expressed was calculated as described in the text. The following ranges of values were obtained: glycine (corrected), 3.86 to 17.39; histidine, 3.20 to 11.63; leucine, 3.33 to 17.00; lysine, 3.70 to 12.20. The correction factor applied to the glycine data is 1.45 (see foot-note 3). The statistical treatment was the same as that in Table IV.

Stimulating substances added to reaction mixture	Relative rates of incorporation (corrected)						His to Leu		His to Lys		Leu to Lys	
	His to Gly		Leu to Gly		Lys to Gly							
	No. of experiments	Average	No. of experiments	Average	No. of experiments	Average	No. of experiments	Average	No. of experiments	Average	No. of experiments	Average
Blank (amino acid mixture alone)*	2	0.825	3	0.89	2	0.70	2	1.00	2	1.18	2	1.16
Blank + iron, 1 γ per ml.*	3	1.17†	2	0.81†	1	0.80†	1	0.96†	1	0.94†	1	0.90†
" + " 1 " " " + plasma filtrate, 0.25 ml. per ml.*	1	1.09†	3	1.15								
Blank + iron, 5.0 γ per ml. + plasma filtrate, 0.25 ml. per ml.*			1	1.03	1	0.74					1	1.39
Blank + iron, 5.0 γ per ml. + plasma filtrate, 0.25 ml. per ml. + fructose-glycine*	1	1.23	1	1.22	1	1.07	1	1.01	1	1.15	1	1.14
Blank + iron, 5.0 γ per ml. + plasma filtrate, 0.25 ml. per ml. + fructose-alanine*	1	1.33	1	1.42	1	1.08	1	0.93	1	1.22	1	1.37
σ		0.36		0.27		0.25		0.05		0.19		0.26
χ^2		2.455		5.531		4.771		2.640		3.856		4.871
P		0.77		0.47		0.44		0.62		0.42		0.30

* The fructose-amino acids were at a concentration of 2×10^{-4} M.

† Reaction time was 2 hours; in all the other experiments it was 4 hours.

of cells, was 8 ml. On this basis and on the assumption of complete mixing, the specific activity of the glycine was reduced to between $8/(8 + 3.2) = 0.7$ and $8/(8 + 7.5) = 0.5$ of that of the glycine added. Some of the unlabeled glycine probably passed out of the cells in the washing prior to their use and this would call for adjusting the dilution factor nearer to 1. The concentrations of histidine, leucine, and lysine in the rabbit plasma are so low (1) that, even if they were concentrated in the reticulocytes to the same degree as glycine, which is unlikely, there would not have been any significant reduction in their specific activities.

DISCUSSION

It is noteworthy that four different kinds of compounds, *i.e.* iron, amino acids, fructose-amino acids, and the unidentified material in plasma, all accelerated the synthesis of heme, amino acid incorporation into globin, the incorporation of histidine, leucine, and lysine into the water-insoluble proteins, and all decreased the incorporation of glycine into the water-insoluble proteins of rabbit reticulocytes. It might have been expected that heme synthesis would be accelerated by iron, but hardly globin synthesis. Conversely, it might have been expected that addition of a mixture of amino acids would accelerate globin synthesis, but hardly, under conditions in which glycine was not limiting, that heme synthesis also would thereby be accelerated.

The labeled heme found in the foregoing experiments must have been synthesized from the labeled glycine, since it could not have become labeled by an exchange process. Heme synthesis and amino acid incorporation into globin are such dissimilar processes that *a priori* there was no reason to expect the rates of the two processes to be even nearly the same. That they were equal is strong presumptive evidence for the exclusion of the following processes as substantial contributors to the labeling of the heme or of the globin: adsorption of labeled amino acids, amino acid exchange in the globin as distinct from its synthesis from amino acids, and contributions from pools of unlabeled intermediates of either heme or of globin or of initially unconjugated and unlabeled heme or globin. If there was not initially a pool of significant size of either heme or of globin, one would not expect this condition to change during the few hours of an experiment *in vitro*. The value of 1 for the ratio of the rate of heme synthesis to that of amino acid incorporation into globin, over a wide range of experimental conditions and rates, leads one to the conclusion that the main process was synthesis of hemoglobin *de novo* from iron and free amino acids as nitrogenous precursors. The evidence argues for a mechanism in the reticulocyte that coordinates and equalizes the rates of heme and globin synthesis.

SUMMARY

In rabbit reticulocytes *in vitro* the rates of heme synthesis from labeled glycine and incorporation of glycine, histidine, leucine, or lysine into the globin and water-insoluble proteins were compared.

1. Iron, amino acids, fructose-amino acids, and plasma filtrate accelerated both heme synthesis and incorporation of each of the four amino acids into globin. They accelerated the incorporation of histidine, leucine, and lysine, but decreased the incorporation of glycine into the water-insoluble proteins in proportion to the stimulation of the other processes.

2. All the findings are in accord in indicating that heme and globin are

synthesized at very nearly the same rate from the amino acid pool without a substantial contribution from unlabeled intermediates of either heme or globin.

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